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Research Article

Improvement of Mouse Parthenogenetic Blastocyst as Primary Colony Source of Parthenogenetic Embryonic Stem Cell (pESC) using CHIR99021

Dwi Budiono[1,5], Ratih Rinendyaputri[2], Ariyani Noviantari[2], Mokhamad Fahrudin[3], Ni Luh Putu Ika Mayasari[4], Vista Budiariati[1,6], Diah Nugrahani Pristihadi[1,3], Noer Muhamad Dliyaul Haq[1], Arief Boediono[3]*

[1]Phd student of Department of Anatomy, Physiology and Pharmacology, Faculty of Veterinary Medicine, IPB University, Bogor, West Java, Indonesia; [2]Center of Biomedic and Basic Technology of Health, National Institute for Health Research and Development (NIHRD) Ministry of Health Republic of Indonesia; [3]Department of Anatomy, Physiology and Pharmacology, Faculty of Veterinary Medicine, IPB University, Bogor, West Java, Indonesia; [4]Department of Animal Diseases and Veterinary Health, Faculty of Veterinary Medicine, IPB University, Bogor, West Java, Indonesia; [5]Veterinary Paramedic Study Program, College of Vocational Studies, IPB University, Bogor, West Java, Indonesia; [6]Faculty of Veterinary Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia.

Abstract | Parthenogenetic blastocysts as a source of parthenogenetic embryonic stem cells (pESC) has low level of blastocyst rate and quality. CHIR99021 is a material that can increase the formation and quality of blastocysts in buffalo through inhibition of glycogen synthase kinase 3. The purpose of this study was to increase the blastocyst rate and quality of parthenogenetic embryos as a source of pESC primary colonies. Fertilized embryos as positive control group. Diploid parthenogenetic embryos were divided into three treatment groups: CHIR99021 0 mmol/l, CHIR99021 0.003 mmol/l, and CHIR99021 0.01 mmol/l. The embryonic stem cell (ESC) primary colonies were cultured from blastocyst stage embryos. The results of this study showed that CHIR99021 0.003 mmol/l was able to significantly increase the blastocyst rate of parthenogenetic embryos ($p<0.05$). The quality of blastocysts produced was also significantly improved ($p<0.05$). Blastocyst rate and quality decreased by increasing CHIR99021 concentration to 0.01 mmol/l. The pESC primary colonies culture showed that blastocysts cultured in CHIR99021 0.003 mmol/l were able to form a pESC primary colony that was similar ($p>0.05$) to fertilized blastocysts to form ESC primary colonies. These results were significantly improved ($p<0.05$) compared to those from blastocysts cultured in CHIR99021 0 mmol/l. The characterization results showed that CHIR99021 0.003 mmol/l showed best pluripotency. The conclusion of this study was that the addition of CHIR99021 0.003 mmol/l is most effective in culture of mice parthenogenetic embryos. This is due to CHIR99021 is able to increase blastocyst rate, blastocyst quality, and formation of pESC colonies with improved quality.

Keywords | Blastocyst, CHIR99021, Embryonic stem cell, Parthenogenetic, Primary colony

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***Correspondence** | Arief Boediono, Department of Anatomy, Physiology and Pharmacology, Faculty of Veterinary Medicine, Bogor Agricultural University, West Java 16680, Indonesia; **Email:** ab@apps.ipb.ac.id
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INTRODUCTION

The difficulties of parthenogenetic embryo production are the low of blastocyst rate (Kuoamo and Kharce, 2015) and the number of inner cell mass (ICM) or quality of blastocysts (Niimura et al., 2002). This certainly affects the formation of parthenogenetic embryonic stem cell (pESC) primary colonies. Some researchers modified the culture system to get a better blastocyst rate and ICM number of blastocysts. Miranda et al. (2016) used a co-culture system to improve the quality and blastocysts rate. Co-culture itself was an embryo culture system that was cultured together with other cell types in the same environment to provide trophic factors that played a role in embryonic development. Contrary to Miranda et al. (2016), Herrick et al. (2017) stated that growth factors which were also produced in the co-culture system was not able to increase the formation of blastocysts and the number of ICM. These conditions indicate that the co-culture system is still being debated among researchers.

A possible chemical compound that can be used to achieve this goal is CHIR99021 (Aparicio et al., 2010; Jeught et al., 2012). This material had been shown to increase the blastocyst rate in bovine embryos (Aparicio et al., 2010). Jeught et al. (2012) stated that CHIR99021 was able to improve the quality of human ICM blastocyst by increasing the expression of the Nanog. The CHIR99021 is a substance that acts as an inhibitor of the glycogen synthase kinase-3 (GSK3) protein. This protein is a serine/ threonin protein kinase that is involved in many signaling in cells. Some of them are PI3K/ PTEN/ Akt/ mTORC1, Race/ Raf/ MEK/ ERK, Wnt/ beta-catenin, Hedgehog, Notch and others (McCubrey et al., 2014). This protein affects the development of the embryo by phosphorylation of β-catenin so that it will be degraded (Aparicio et al., 2010). β-catenin is an intracellular protein which affect cell attachment and the process of gene transcription in the *Wnt* signaling pathway (Valenta et al., 2012). Degradation of β-catenin can disturb the embryo development by inhibition of *Wnt* gene transcription (Aparicio et al., 2010). CHIR99021 can inhibit the degradation of β-catenin by GSK3 so that it will increase blastocyst rate and quality.

The purpose of this study was to significantly increase the blastocyst rate and blastocyst quality of parthenogenetic embryos of mice using CHIR99021 as a source of pESC primary colonies.

MATERIALS AND METHODS

Ethical approval

This study was approved by the Animal Ethics Commission of the Faculty of Veterinary Medicine, IPB University and was given the animal ethics certificate number 124/ KEH/ SKE/ IV/ 2019.

Research animals

Three months old male and female mice DDY strain were used in this experiment. The mice were kept indoors with a temperature of 22-25 °C, humidity of 70%, and lighting for 12 hours. Mice were given food and water *ad libitum*. Female mice were injected with pregnant mare serum gonadotrophin (PMSG) (Intervet®) 5 international units (I.U), intraperitoneally. Human chorionic gonadotropin (hCG) (Intervet®) 5 I.U injection was performed 48 hours after PMSG injection, intraperitoneally (Budiono et al., 2018). The superovulated mice were then grouped. The first group was used for parthenogenesis. The second group was used to produce fertilized zygotes by mating with male and female ratio of 1: 1.

Parthenogenesis activation and culture

Superovulated and mated mice were sacrificed 14 hours after HCG injection. Reproductive organs were placed into a petri dish containing Dulbecco's phosphate buffered saline (DPBS, Sigma-Aldrich®). Cumulus oocyte complex (COC) and zygotes were obtained by tearing up the ampulla of the fallopian tubes. The COC were then placed into hyaluronidase 0.25% (Sigma-Aldrich®) and then incubated at 37 °C for 5 minutes until the cumulus cells stretched, then followed by pipetting (Nagy et al., 2003).

The embryo medium used in the study was a modification of the modified rat 1-cell embryo culture medium (mR1ECM) made by Miyoshi et al. (1995). Modifications were carried out by replacing polyvinyl alcohol with bovine serum albumin (BSA). The activation medium consists of mR1ECM, strontium chloride ($SrCl_2$) 10 mmol/l (Sigma-Aldrich®) and cytokalasin B (cyt B) 0.0005% (Sigma-Aldrich®). Activation was carried out for 6 hours in the incubator at 37°C and 5% CO_2 (Murti et al., 2010).

The fertilized zygotes cultured using mR1ECM were used as positive control. The diploid parthenogenetic zygotes were divided into three treatment groups based on their culture conditions. Group CHIR99021 0 mmol/l was cultured using mR1ECM + CHIR99021 0 mmol/l (Sigma-Aldrich®) medium. Group CHIR99021 0.003 mmol/l was cultured using mR1ECM + CHIR99021 0.003 mmol/l. Group CHIR99021 0.01 mmol/l was cultured using mR1ECM + CHIR99021 0.01 mmol/l. Embryo cultures were carried out for 4 days until blastocysts stage in the incubator at 37°C and 5% CO_2.

Embryos development data were obtained using cleavage rate, morula rate, and blastocyst rate. The cleavage rate is the average value of the number of cleavage embryos

divided by the total number of zygotes. The morula rate is the average value of the number of morula embryos divided by the total number of zygotes. Blastocyst rate is the average value of the number of embryos that makes up a blastocyst divided by the total number of zygotes.

Blastocysts morphometry

Blastocysts morphometry was measured using imageJ version 1.390 software (Wayne Rasband, National Institutes of Health, USA).

Parthenogenetic embryonic stem cells (pESC) and embryonic stem cells (ESC) primary colony culture

The primary colonies of pESC and ESC were cultured using the same medium (Budiono et al., 2018). Fertilized blastocysts were used as positive control of primary colony sources. Parthenogenetic blastosis used as primary colony source were derived from parthenogenetic embryos cultured in mR1ECM medium with the addition of CHIR99021 0 mmol/l and CHIR99021 0.003 mmol/l. Each blastocyst group was placed into pronase 0.25% (Sigma-Aldrich®) for 5 minutes at 37°C. This process aimed to lyse the zona pellucida which covered the blastocyst. Blastocysts without the zona pellucida were each cultured on a petri dish containing a REF feeder cell. Culture was carried out in an incubator at 37°C and 5% CO2. The culture medium was replaced every 2 days. Culture was carried out for 7 days then pluripotency characterization was performed using staining of alkaline phosphatase (ALP), immunocytochemical (ICC) kit of *Oct4*, *Nanog*, and SSEA1.

Attachment rate (AR) is the number of blastocysts attached to the feeder cell divided by the total number of blastocysts cultured. Primary rate (PC) is the number of blastocysts capable of forming primary colonies divided by the number of blastocysts cultured.

Immunocytochemistry (ICC)

Immunocytochemistry (ICC) was used to characterize pluripotency of all groups using the anti-*Oct4* (Santa Cruz sc®), anti-*Nanog* (Santa Cruz sc®), and anti-SSEA1 (Santa Cruz sc®) antibodies. HRP-conjugated antibody as secondary antibodies and other reagents used Trekkie Universal Link (Star Trek Universal HRP Detection Kit Biocare®). The staining procedure was done according to company guidelines which were modified according to research conducted by Rinendyaputri et al. (2018).

Alkaline phosphatase staining

The 7th day colonies were washed using DPBS 3 times. Colonies were fixed using paraformaldehyde 4% for 2 minutes. Colonies that were fixed then washed using mili-Q and then stained with naphtol AS-MX phosphatase 200 µg/ml (Sigma-Aldrich®) and fast red TR salt 1 g/ml (Sigma-Aldrich®) in tris buffer 100 mmol/l. Incubation was carried out for 30 minutes at room temperature. Colonies that have been stained were then washed using milli-Q 2 times (Budiono et al., 2018).

Data analysis

Qualitative data were presented in form of images. Quantitative data were calculated using one-way analysis of variance (ANOVA) (confidence level of 95%) with Duncan post test. Results were presented in the mean ± standard error.

RESULTS AND DISCUSSION

Embryo development

Embryos development data are presented in Table 1. Results showed that the cleavage rate did not differ significantly between treatments ($p>0.05$). Morula rate from CHIR99021 0.003 mmol/l group was not significantly different ($p>0.05$) from CHIR99021 0 mmol/l and CHIR99021 0.01 mmol/l group. This value (morula rate) was significantly different ($p<0.05$) when compared to positive control group. The blastocysts rate from CHIR99021 0.003 mmol/l group had the highest value ($p<0.05$). The value of blastocyst rate parthenogenetic embryo decreased significantly ($p<0.05$) along with the increase in the concentration of CHIR99021 to 0.01 mmol/l. Blastocyst rate of CHIR99021 0 mmol/l and CHIR99021 0.003 mmol/l group were significantly different ($p<0.05$) when compared to the positive control group and CHIR99021 0.01 mmol/l group.

Blastocysts morphometry

The blastocyst morphometry of this study are shown in Table 2. The blastocyst diameter of CHIR99021 0.003 mmol/l group was significantly higher ($p<0.05$) compared to CHIR99021 0 mmol/l group ($p<0.05$). This value (blastocyst diameter) did not differ significantly from control and CHIR99021 0.01 mmol/l group ($p>0.05$). The inner cell mass (ICM) diameter from CHIR99021 0.003 mmol/l group was significantly larger ($p<0.05$) when compared to CHIR99021 0 mmol/l and CHIR99021 0.01 mmol/l group. The ICM diameter of this group was not significantly different ($p>0.05$) when compared to positive control group. The ICM diameter of CHIR99021 0 mmol/l and CHIR99021 0.01 mmol/l group was not significantly different ($p>0.05$).

Primary colonies culture

Primary colony culture data are presented in Table 3. The results showed that the AR value of the CHIR99021 0.003 mmol/l group was not significantly different ($p>0.05$) compared to CHIR99021 0 mmol/l and positive control

Table 1: Development of parthenogenetic and fertilization embryos, *in vitro* conditions.

Treatment Groups	N	Cleavage rate, n (%)	Morula rate, n (%)	Blastocyst rate, n (%)
Control	99	94 (94.47±1.64)[a]	63 (63.37±4.84)[a]	50 (50.63±3.65)[a]
CHIR99021 0 mmol/l	131	120 (91.43±3.23)[a]	94 (72.08±4.89)[abc]	82 (62.62±2.97)[b]
CHIR99021 0.003 mmol/l	70	62 (88.22±4.89)[a]	56 (80.28±1.80)[c]	49 (70.13±1.01)[c]
CHIR99021 0.01 mmol/l	98	90 (86.47±8.60)[a]	73 (75.62±7.32)[bc]	54 (55.81±3.91)[a]

N: Number of zygot; n: Number of embryo (cleavage, morula, blastocyst); Values with different letters within a column indicates significant differences ($p < 0.05$).

group. The AR value of the positive control group was significantly different from the CHIR99021 0 mmol/l group ($p<0.05$). Primary rate (PC) CHIR99021 0.003 mmol/l group was significantly higher ($p<0.05$) than CHIR99021 0 mmol/l group. The Primary rate (PC) of CHIR99021 0.003 mmol/l group was not significantly different ($p>0.05$) from positive control group.

Table 2: Morphometry of blastocysts dan inner cell mass (ICM).

Treatment groups	n	Blastocyst diameter (µm)	ICM diameter (µm)
Control	26	98.31±6.90[c]	24.47±2.66[b]
CHIR99021 0 mmol/l	29	90.11±6.73[a]	21.32±2.35[a]
CHIR99021 0.003 mmol/l	31	95.38±8.63[bc]	23.58±2.81[b]
CHIR99021 0.01 mmol/l	26	92.30±5.69[ab]	22.04±1.45[a]

n: Number of blastocysts; Values with different letters within a column indicates significant differences ($p < 0.05$).

Table 3: Attachment rate (AR) and primary rate (PC) of blastocysts cultured in feeder cells.

Treatment groups	n	Attachment rate, n (%)	Primary rate, n (%)
Control	96	94 (97.91±2.41)[b]	83 (88.66±4.37)[b]
CHIR99021 0 mmol/l	57	46 (78.20±14.97)[a]	34 (56.93±16.64)[a]
CHIR99021 0.003 mmol/l	100	94 (92.56±3.97)[ab]	80 (78.01±7.75)[b]

n: Number of blastocysts; Values with different letters within a column indicates significant differences ($p < 0.05$).

Immunocytochemistry (ICC)

The result of ICC staining present in Table 4. The result indicated that the primary colonies of all groups expressed pluripotency characteristics. Positive result was shown with brown primary colonies (Fig. 1A). Negative result was shown with colorless primary colonies (Fig. 1B).

Table 4: Immunocytochemistry of primary colonies.

Treatment groups	*Oct4*	*Nanog*	SSEA1
Control	+	+	+
CHIR99021 0 mmol/l	+	+	+
CHIR99021 0.003 mmol/l	+	+	+

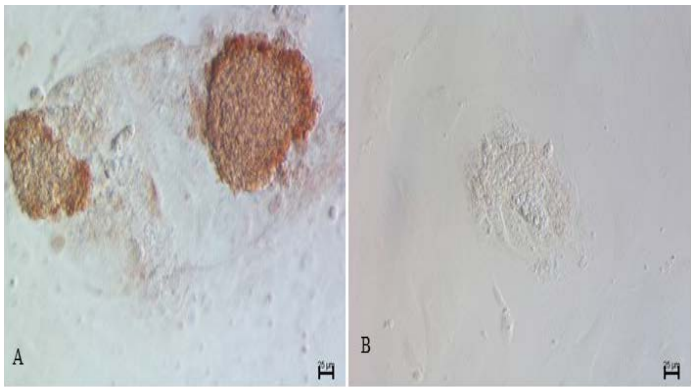

Figure 1: Immunocytochemistry staining of the 7th day primary colonies of culture. (A) Immunocytochemistry positive. (B) Immunocytochemistry negative. Bar = 25µm.

Alkaline phosphatase (ALP) staining

Alkaline phosphatase staining results showed that the primary colony of CHIR99021 0.003 mmol/l group (Fig. 2C) expressed stronger ALP activity when compared to the primary colony of control (Fig. 2A) and CHIR99021 0 mmol/l group (Fig. 2B). All parts of the primary colonies of CHIR99021 0.003 mmol/l group were red which indicated ALP activity (Fig. 2C). There were some of the non-red at the primary colonies of CHIR99021 0 mmol/l group (blue circles) which indicated no ALP activity (Fig. 2B). Primary colonies of positive control group showed ALP enzyme activity found in all parts of the colony (Fig. 2A), but there were some colonies that showed weak ALP enzyme activity shown in faded red (blue arrows).

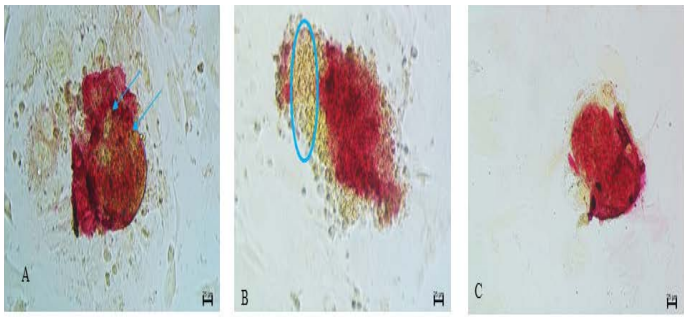

Figure 2: Alkaline phosphatase (ALP) staining of primary colony on 7th day of culture. (A) Control; (B) CHIR99021 0 mmol/l; (C) CHIR99021 0.003 mmol/l group. Blue arrow: weak ALP expression; Clue circle: No ALP expression. Bar = 25µm.

This study is the basic study to carry out mice parthenogenetic embryo culture using mR1ECM medium added with CHIR99021 as GSK3 inhibitors. The result of this study is improved when compared with study which conducted by Popova et al. (2011). The results of their study showed that the blastocyst rate of parthenogenetic embryos cultured at mR1ECM without CHIR99021 was 66.1%, where the blastocyst rate produced cultured using the addition of CHIR99021 in this study was 70.13%. This shows that CHIR99021 is effective when used in parthenogenetic embryos in mice with a concentration of 0.003 mmol/l.

GSK3 protein is involved in the *Wnt* pathway by phosphorporating β-catenin. CHIR99021 specifically inhibits GSK3 to phosphorylate β-catenin (Aparicio et al. 2010; Kwon et al., 2019). Furthermore, the result shows that the addition of CHIR99021 0.003 mmol/l in parthenogenetic embryo culture which starts from the early stages of embryonic development increased the blastocyst rate in this study. This demonstrates that the occurrence of GSK3 inhibition in the phosphorylation of β-catenin parthenogenetic embryo occurs as well. This causes β-catenin passes through the *Wnt* pathway to be concentrated in the nucleus. High β-catenin in the nucleus causes the transcription of *Wnt* genes to form blastocysts (Aparicio et al. 2010). In addition, GSK3 is also suspected to be strongly involved in the process of apoptosis in embryonic development. This is the reason that embryos cultured using CHIR99021 showed a decrease in apoptosis (Kwon et al., 2019). In general, apoptosis in cells is induced by p53 after DNA damage (Williams and Schumacher, 2016) and GSK3 then activates the caspases pathway and induces apoptotic pathway (Watcharasit et al., 2002). This is reinforced by the opinion of Wang et al. (2015) that p53 dependent apoptosis can be reduced using CHIR99021.

According to the findings of this study, the blastocyst rate decreases with increasing CHIR99021 concentration to 0.01 mmol/l ($p<0.05$). Research conducted by Tribulo et al. (2017) showed that bovine embryos cultured in CHIR99021 0.01 mmol/l had decreased ability to become blastocysts. This because GSK3 involve in many signaling pathway (McCubrey et al., 2014). The mechanism by which excessive amounts of β-catenin cause a decrease in blastocyst rate is unclear.

The quality of parthenogenetic blastocysts in this study were assessed based on the diameter of the blastocysts and ICM. Almagor et al. (2016) reported that blastocyst and ICM diameter were correlated positively with pregnancy rate. This indicates that blastocyst diameter and ICM may be used to determine blastocyst quality. The results showed that CHIR99021 with a concentration of 0.003 mmol/l improved the quality of the parthenogenetic blastocysts. According to Harris et al. (2013), blastocysts produced from embryos cultured in a medium containing CHIR99021 enhanced the number of trophectoderm (TE) and ICM. The mechanism of TE number improvement occured due to an increase in transcription of *Sox-2* gene. *Sox-2* is the member of *Wnt* gene which plays a role in TE formation. The TE improvement led to increase of blastocyst diameter in this study. The increase in ICM size in this study occurred due to an increase in the number of pluripotent blastomers when the embryo was in the blastocyst phase (Tribulo et al., 2017). According to Mori et al. (2002), there were positive correlations between the cell number and blastocyst diameter. The larger the diameter of the blastocyst, the greater the number of cells. This occured because the inhibition of GSK3 caused an increase in the synthesis of pluripotency transcription factors such as *Nanog, Tbx3*, and *c-Myc* (Sanchez-Ripoll et al., 2013). The results of this study recommend the use of CHIR99021 with a concentration of 0.003 mmol/l as a supplement in the culture of mice parthenogenetic embryos. This is due to CHIR99021 with a concentration of 0.003 mmol/l gives a better result in blastocyst rate and quality.

The addition of CHIR99021 0.003 mmol/l in culture medium in this study improved the formation of primary colony. According to the results of the study by Muzaffar et al. (2012) and Shah et al. (2015) that parthenogenetic embryonic stem cell primary colonies culture showed lower primary rate values when compared to fertilized blastocysts. This study showed that parthenogenetically blastocysts which were cultured in CHIR99021 0.003 mmol/l could form primary colony as good as fertilized blastocysts. This condition did not occur at parthenogenetically blastocysts which was cultured in CHIR99021 0 mmol/l. This condition occured because of an increase in the quality of parthenogenetic blastocysts. According to Cheong et al. (2015), the greater the blastocysts quality, the greater the ability to form ESC. The results of their study were in accordance with the results of this study where the quality of parthenogenetic blastocyst cultured using CHIR99021 0.003 mmol/l was better than those cultured with CHIR99021 0 mmol/l and was not significantly different than control.

The primary colony characterization of this study was carried out ALP staining and ICC staining using anti-*Oct-4*, anti-*Nanog*, and anti-SSEA1 antibodies. Immunocytochemistry (ICC) staining results generally showed that primary colonies of all groups indicated positive result for all antibody. This result indicated that all group had good pluripotency primary colony. Good pESC would express *Oct3 / 4, Nanog*, and SSEA1 (Varrault et al., 2018). The expression showed that the pESC was undifferentiated and pluripotent. Zhao et al. (2012) stated that *Oct4, Nanog*, and SSEA1 were the main pluripotent markers that determine

the level of ESC pluripotence in murine.

Alkaline phosphatase (ALP) is a glycoprotein enzyme which catalyzes the hydrolysis of phosphate monoester (Plotnikov et al., 2017). This enzyme is expressed by inner cell mass (ICM), primordial germ cells (PGC), embryonic stem cells (ESC), and induced pluripotent stem cells (iPS). Previous studies had shown that mice pESC expressed ALP enzymes as well as ESC (Liu et al., 2016). The pESC was cultured in other species such as humans (Yu et al., 2015), primates (Wei et al., 2011), pigs (Cheong et al., 2015), and buffalo (Shah et al., 2015) also showed strong ALP expressions. ALP enzyme expression in the CHIR99021 0.003 mmol/l group in this study was stronger than the other groups. This condition occured because CHIR99021 0.003 mmol/l group expressed higher pluripotency transcription factors (Sanchez-Ripoll et al., 2013) thus, ALP expression was also higher. According to Štefková et al. (2015), high ALP expression showed that the cell was in pluripotent condition. The ALP expression will decrease along with the differentiation experienced by pluripotent stem cells. This showed that CHIR99021 0.003 mmol/l in parthenogenetic embryo culture could improved the quality of the primary colonies with strong ALP expression.

CONCLUSIONS AND RECOMMENDATIONS

This research attempts to inhibit β-catenin phosphorylation by inhibiting GSK3 in the early parthenogenetic embryo development stage. The culture condition is expected to produce more blastocyst rate when compared to culture without CHIR99021. There are no studies that have reported the effectiveness of CHIR99021 in increasing the blastocyst rate and quality of parthenogenetic embryos in mice. This is the basic research that studied the use of CHIR99021 in the culture system of mice parthenogenetic embryos. This study also conducted pESC primary colony cultures originating from parthenogenetic blastocysts cultured using CHIR99021.

The study concluded that the addition of CHIR99021 0.003 mmol/l is the most effective in culture of mice parthenogenetic embryos. This is because CHIR99021 is able to increase blastocyst rate, blastocyst quality, and formation of pESC colonies with good quality.

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NOVELTY STATEMENT

This article is to reveal the mechanism of action of CHIR99021 to increase the blastocyst rate and quality of the blastocyst in mice parthenogenetic embryos. Previous research applied CHIR99021 to increase blastocyst rate and blastocyst quality in buffalo fertilized embryos. This study also proved that good quality of parthenogenetic blastocysts would improve the quality of pESC primary colonies.

AUTHOR'S CONTRIBUTION

Dwi Budiono: Concenption and design of studi, Acquisition of data, Data analysis and/or interpretation, Drafting of manuscript and/or critical revision, Approval of final version of manuscript. Ratih Rinendyaputri: Acquisition of data, Data analysis and/or interpretation, Methodology, Drafting of manuscript and/or critical revision, Approval of final version of manuscript. Ariyani Noviantari: Acquisition of data, Data analysis and/or interpretation, Resources, Drafting of manuscript and/or critical revision, Approval of final version of manuscript. Mokhamad Fahrudin: Concenption and design of studi, Supervision, Drafting of manuscript and/or critical revision, Approval of final version of manuscript. Ni Luh Putu Ika Mayasari: Concenption and design of studi, Drafting of manuscript and/or critical revision, Validation, Approval of final version of manuscript. Vista Budiariati: Drafting of manuscript and/or critical revision, Approval of final version of manuscript. Diah Nugrahani Pristihadi: Drafting of manuscript and/or critical revision, Approval of final version of manuscript. Noer Muhamad Dliyaul Haq: Drafting of manuscript and/or critical revision, Software. Arief Boediono: Concenption and design of studi, Supervision, Acquisition of data, Data analysis and/or interpretation, Drafting of manuscript and/or critical revision, Approval of final version of manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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